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*Published in:*  
Drug Discovery Today

*DOI:*  
[10.1016/j.drudis.2019.02.011](https://doi.org/10.1016/j.drudis.2019.02.011)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2019

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Liu, B., Saber, A., & Haisma, H. J. (2019). CRISPR/Cas9: A powerful tool for identification of new targets for cancer treatment. *Drug Discovery Today*, 24(4), 955-970. <https://doi.org/10.1016/j.drudis.2019.02.011>

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*Teaser This review provides the latest findings regarding the application of CRISPR/Cas9 for the identification of new therapeutic targets and associated major challenges in cancer treatment.*



# CRISPR/Cas9: a powerful tool for identification of new targets for cancer treatment

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Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9), as a powerful genome-editing tool, has revolutionized genetic engineering. It is widely used to investigate the molecular basis of different cancer types. In this review, we present an overview of recent studies in which CRISPR/Cas9 has been used for the identification of potential molecular targets. Based on the collected data, we suggest here that CRISPR/Cas9 is an effective system to distinguish between mutant and wild-type alleles in cancer. We show that several new potential therapeutic targets, such as *CD38*, *CXCR2*, *MASTL*, and *RBX2*, as well as several noncoding (nc)RNAs have been identified using CRISPR/Cas9 technology. We also discuss the obstacles and challenges that we face for using CRISPR/Cas9 as a therapeutic.

## Introduction

The accumulation of genetic mutations in cells over time leads to cancer. In addition, certain gene changes, such as driver mutations in *TP53*, *EGFR*, *KRAS*, *BRAF*, *HER2*, and *MET*, can make a cell cancerous. Treatment strategies are conventionally based on histological subtypes. However, in addition to the conventional histological classifications, each cancer type can now be subdivided into various molecular subtypes that have a crucial role in the treatment decision-making process. Each molecular subtype is treated differently and clinicians can also predict treatment outcomes and patient survival. For instance, patients with lung cancer with *EGFR*-activating mutations are treated with different types of tyrosine kinase inhibitor (TKI), such as gefitinib, erlotinib, or afatinib, depending on the mutation. Yet, resistance to the TKIs inevitably emerges either by DNA mutations or/and metabolic changes. As a result, treatment strategies are modified based on the new molecular signature. However, eventually, the tumor cells do not respond to any treatment [1]. Therefore, identification of new therapeutic targets to improve patient survival and clinical outcomes is crucial [2,3].

In recent years, CRISPR/Cas9 has significantly influenced the field of molecular biology and gene therapy. Solid tumors are the most common type of tumors, but less progress has been made

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**Hidde Haisma** was awarded an MSc in medical biology at the University of Utrecht, The Netherlands in 1983; he then worked as a scientist at Centocor Inc. Malvern, PA, USA (1985–1986), Dana Farber Cancer Institute, Brigham and Women's Hospital Boston MA, USA (1986–1987), and as an instructor at Harvard Medical School, Boston MA, USA (1986–1987). In 1987, he was awarded his PhD by the University of Utrecht, The Netherlands for his thesis entitled 'Monoclonal antibodies and cancer'. From 1987 to 1997, he worked as a scientist in the Section for Experimental Therapy of the Department of Medical Oncology, University Hospital of Amsterdam, The Netherlands, where, in 1997, he was appointed assistant professor/section leader of the Division of Gene Therapy. In 2000, he became a full professor at the University of Groningen, The Netherlands. Haisma's research is devoted to the development of genetic medicines for therapeutic intervention of gene expression in patients. He is member of the editorial board of several journals, has co-authored over 150 publications, and holds several patents. Haisma is the former president and member of the board of The Dutch Society of Gene and Cell Therapy. In addition, he is member of the Gene and Cell Doping Expert Group of the World Anti-Doping Agency (WADA).



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for gene therapy-based treatment compared with nonsolid tumors, such as leukemia. However, this situation is rapidly changing with developments in CRISPR/Cas9. There is a growing amount of promising preclinical data showing CRISPR/Cas9 to be an effective tool to specifically target cancer cells and suppress tumor growth [4–6]. This could lead to the discovery of novel molecular targets for cancer treatment.

There is an increasing number of clinical trials utilizing CRISPR/Cas9 technology to treat cancers of different origin (Table 1). Most of these trials are based on genetically engineered T cells for cancer immunotherapy, rather than targeting a specific gene in the tumor cells themselves. One of the main problems associated with the direct targeting of cancer is the lack of an effective and safe delivery method that can be used in patients. Tumor heterogeneity is another issue that might be a challenge because tumors usually comprise different subclones (i.e., intratumor heterogeneity) [7–10]. Thus, even with the right delivery system, outgrowth of a minor subclone can emerge and the treatment would no longer be effective. Nonetheless, identification of different major subclones before treatment and recruitment of multiple Cas9/guide (g)RNA might be an option to minimize relapse in patients.

Here, we provide an overview of studies in which CRISPR/Cas9 has been utilized for the identification of potential therapeutic targets in some of the most frequent solid tumors, including lung, breast, brain, liver, and colorectal cancer. We discuss potential candidates for therapy that are either highly expressed or activated in different cancer types, because they are more convenient to inhibit or disrupt. We end by presenting recent advances in, and different delivery methods for, CRISPR/Cas9.

### CRISPR/Cas9 gene-editing technology

CRISPR/Cas9 is a recently discovered, powerful gene-editing tool derived from a prokaryotic defense system [11–14]. This technology has enabled researchers to edit the genome of eukaryotic cells more precisely and efficiently compared with previous methods,

such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) [15].

#### The structure of CRISPR/Cas9

The structure of CRISPR/Cas9 has comprehensively been described elsewhere [15–17]. The CRISPR/Cas9 system has three components; a single guide RNA (sgRNA), which is specific to a target sequence of DNA; Cas9 protein with DNA endonuclease activity; and a tracrRNA that interacts with Cas9 (Fig. 1). The gRNA (approximately 20 base pairs in length) binds to the target site in the genome and directs the Cas9 protein. The Cas9 protein is a RNA-guided nuclease that was discovered in the CRISPR type II adaptive immunity system of *Streptococcus pyogenes* and it is responsible for cleaving double-strand DNA [17].

#### gRNAs and their specificity

Several factors, such as sequence, length, and secondary structure, of gRNAs can influence their efficiency and specificity [18,19]. In addition, the efficiency of the CRISPR/Cas9 complex can be influenced by other factors, including the genomic locus of the target, chromatin accessibility, nucleosomes, and other components around gRNA-binding sites [19]. The gRNA sequence has a crucial role in the efficiency, specificity, and accuracy of CRISPR/Cas9-mediated genome editing. The first 10–12 nucleotides at the 3' end of gRNA, immediately adjacent to a protospacer adjacent motif (PAM), called the 'seed sequence', bind to the target sequence and determine the specificity [18,20]. Truncated gRNAs with shorter complementary nucleotides (<20) can reduce off-target effects by 5000-fold without sacrificing on-target efficiency [21]. Moreover, extending the gRNA duplex by 5 base pairs can significantly improve the knockout efficiency [22].

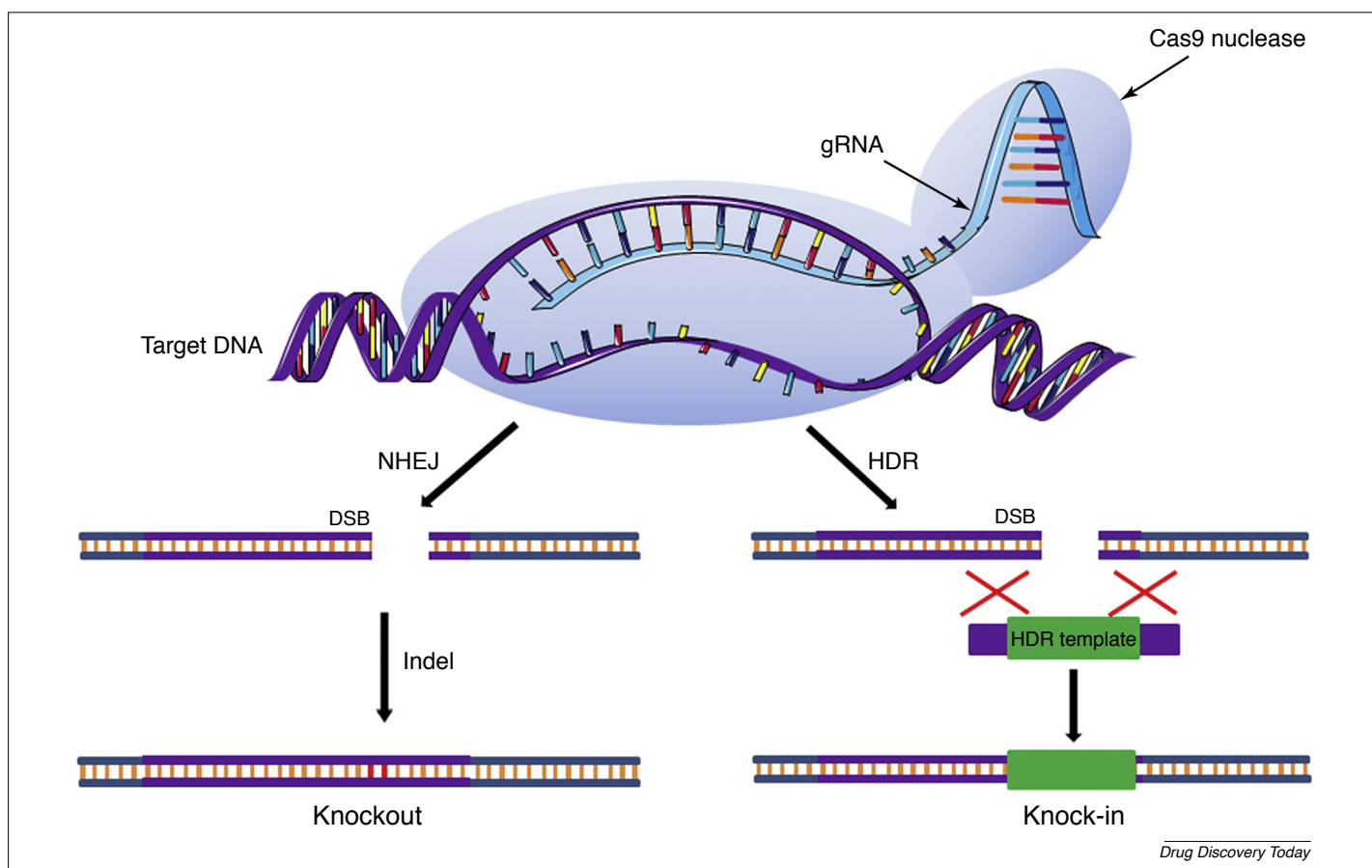
#### CRISPR-associated nucleases

Different versions of CRISPR-associated nucleases are currently under development, greatly expanding the CRISPR-based toolbox for genome editing (Table 2). Cpf1 is an RNA-guided endonuclease that belongs to the class 2 CRISPR-Cas system, the same as Cas9

TABLE 1

#### Phase I/II clinical trials that use CRISPR/Cas9 genome-editing technologies

Condition	Target	Interventions	Phase	Status	Clinical Trials Gov Identifier
Solid tumor, adult	PD-1 and TCR	Anti-mesothelin CAR-T cells	I	Active	NCT03545815
Melanoma; synovial sarcoma; liposarcoma	TCRendo and PD-1	NY-ESO-1; drug: cyclophosphamide, fludarabine	I	Active	NCT03399448
Gastrointestinal cancer	CISH, inactivated TIL	Drug: cyclophosphamide, fludarabine, aldesleukin	I	–	NCT03538613
Human papillomavirus-related malignant neoplasm	HPV-related cervical intraepithelial neoplasia	Biological: TALEN, CRISPR/Cas9	I	–	NCT03057912
Gastrointestinal infection	Host factors of norovirus	Duodenal biopsy; saliva	–	Active	NCT03342547
B cell leukemia; B cell lymphoma	CD19 and CD20 or CD22 CAR-T	Universal dual-specificity CD19 and CD20 or CD22 CAR-T Cells	I/II	Active	NCT03398967
Leukemia; lymphoma	Relapsed or Refractory CD191	UCART019	I/II	Active	NCT03166878
Stage IV gastric carcinoma; Stage IV nasopharyngeal carcinoma; Stage IV T cell lymphoma	–	Drug: fludarabine, cyclophosphamide, interleukin-2	I/II	Active	NCT03044743

**FIGURE 1**

Schematic picture of genome editing mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9), and DNA repairing. The Cas9 protein, which is guided by a desired single-strand guide RNA (gRNA), cuts the double-stranded DNA and makes a double strand break (DSB). Subsequently, DNA repair occurs through either the nonhomologous end joining (NHEJ) or the homology-directed repair (HDR) pathways.

[23]. However, Cpf1 has different features compared with Cas9. For example, it has only one single nuclease domain and a shorter gRNA. Creating staggered cuts is one of the main characteristic features of Cpf1. This type of cut is important for introducing exogenous DNA into the genome by the homology directed repair (HDR) pathway. In addition, Cpf1 has both endoribonuclease and endonuclease activities, which is unique for a nuclease [24]. These properties make Cpf1 both a complex and effective genome-editing tool for gene targeting and gene silencing. C2C2 is another member of class 2 type VI-A CRISPR-Cas and was found in *Leptotrichia shahii*, where it protects the bacterium against RNA phages. C2C2 can be used to target and regulate RNAs. It has two RNase catalytic pockets with dual RNase activities, which can be recruited for the identification of cellular transcripts [25]. The structure and function of C2C2 is unique and provides a novel tool for RNA manipulation [25–27].

Precisely editing a single base in the genome without introducing double-stranded breaks (DSBs) was a longstanding goal that, with engineered Cas9 base editors, is now possible. The 'base editors' comprise fusions of a dead Cas9 domain and a cytidine deaminase enzyme that is able to convert GC to AT without introducing DSBs [28]. Recently, researchers created a Cas9 fused with a transfer RNA adenosine deaminase that can mediate conversion of AT to GC [29]. These base editors are valuable tools for repairing disease-related mutations.

#### Advantages of CRISPR/Cas9 over ZFN and TALENs

The CRISPR/Cas9 system has several advantages over ZFN and TALENs in terms of its simplicity, flexibility, and affordability. The most important difference is that the CRISPR system relies on RNA–DNA recognition, rather than on the protein–DNA-binding mechanism [11,17,30]. Thus, it is more 'doable' and easier to construct a customized CRISPR/Cas9 complex by only changing the gRNA sequence instead of engineering a new protein. The target sequence needs to be immediately upstream of a PAM sequence (5'-NGG-3') [17], because the latter is essential for target recognition by Cas9. This short sequence occurs approximately once every eight base pairs in the human genome, which makes it possible to design several gRNAs for one specific target gene [31].

#### Targeting cancer-related genes and identification of potential therapeutic targets in solid tumors

CRISPR/Cas9 is routinely used in research laboratories because of its simplicity and efficiency. In addition, the cost of this gene-editing tool is reducing daily. The CRISPR/Cas9 gene-editing technology helped researchers to identify the role of different genes in cancer; for instance whether they function as oncogenes or tumor genes [32–36]. Several groups generated *in vitro* and *in vivo* knockout models to study the molecular basis of different cancer types [37–39]. CRISPR/Cas9 is also widely used for inducing specific mutations in certain genes to explore the potential causative

TABLE 2

## CRISPR-associated nucleases

Class	Name	Advantages	Applications	Refs
Naturally Cas9	SpCas9	Most common used	Gene editing	[17,23,200,201]
	SaCas9	1 kb smaller than SpCas9		
	StCas9	Differ in PAM sequence		
	CjCas9			
	FnCas9		Potentially useful untapped nucleases	
	CasX	Most compact naturally CRISPR variant		
	CasY			
	Cas12a (Cpf1)	Smaller than SpCas9; gRNA shorter than SpCas9	More economical to produce	
Engineered Cas9 variants	Cas9 nickases	Variants nick a single DNA strand rather than DSB	High fidelity	[202,203]
	eSpCas9	Enhanced nuclease	High specificity and low off-target rate	
Base editors	BE1/2/3; ABEs	Complete range of nucleotide exchanges without DSBs	Gene writing	[28,29]
RNA editors	Cas13a/Cas13b/Cas13d	Targeting RNA rather than DNA	RNA editing	[26,200,204]

role of these mutations in disease development. In addition, CRISPR barcoding technology can be used to investigate tumor heterogeneity [40,41]. Moreover, genome-wide CRISPR/Cas9 screening is frequently used to identify potential therapeutic targets in different cancers. Here, we mainly focus on new potential molecular targets that have been identified by using CRISPR/Cas9 in some of the most frequent solid tumors, including lung, breast, brain, liver, and colorectal cancers.

### Lung cancer

Lung cancer is the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) subtypes account for ~85% of all lung cancers [42]. The identification of specific genetic aberrations is important to choose the appropriate treatment strategy, particularly in patients with adenocarcinoma. Currently, several molecular targets, such as EGFR, BRAF, ALK-EML4, and cMET, are clinically available for the treatment of NSCLC. However, treatment options are limited by the number of molecular targets and by emerging drug resistance [1,42]. In recent years, CRISPR/Cas9-based *in vitro* and *in vivo* studies of lung cancer have identified new treatment strategies and potential therapeutic targets.

Targeting the mutant version of certain genes using the CRISPR/Cas9 gene-editing system can specifically target mutant cancer cells, but not normal cells. Targeting the mutant version of the EGFR gene (L858R) resulted in the selective elimination of mutant cells and reduced cell proliferation both *in vitro* and *in vivo* [43]. In another study, Koo *et al.* selectively abrogated EGFR mutant alleles (L858R) in a NSCLC cell line (H1975) using an adenovirus (AdV) vector that resulted in cancer cell death and significantly reduced tumor size *in vivo* [4]. These findings underscore the potential of CRISPR-based therapeutics in tumor-specific targeted therapy and in distinguishing normal from mutant tumor cells.

Approximately 30% of patients with lung cancer have somatic activating KRAS mutations. Currently, there is no effective treatment for KRAS mutant lung tumors, which are considered as undruggable. CRISPR-based deletion of murine *Kras* in two differ-

ent *Kras*<sup>G12D/1</sup>/*p53*<sup>-/-</sup> lung cancer cell lines resulted in a significant reduction in cell proliferation, but the cells were still viable and sustained their ability to form tumors *in vivo*. Transcriptome sequencing revealed a substantially higher expression of Fas receptor in the knockout cells. Interestingly, an activating Fas receptor antibody selectively induced apoptosis in these *Kras*<sup>-/-</sup> lung cancer cells [44]. Oncogenic Ras can inhibit Fas ligand-mediated apoptosis through downregulation of Fas [45]; therefore, simultaneous inhibition of KRAS and activation of FAS might be an effective therapeutic approach against KRAS-driven lung cancer tumors.

CD38 is a glycoprotein that functions as both an ADP-ribosyl cyclase and a NAD glycohydrolase. Its expression level is negatively associated with poor prognosis in patients with chronic lymphocytic leukemia and it is used as a therapeutic target in multiple myeloma [46,47]. However, its role in solid tumors, such as lung cancer, is not clear. CRISPR-based deletion of CD38 in a lung adenocarcinoma cell line (A549) resulted in substantial suppression of cell growth and invasion *in vitro* and in xenografts in mice, suggesting CD38 as a potential target in lung cancer. Further investigations unraveled an elevated level of CD38 in 93% (27/29) of lung cancer cell lines and 40% (11/27) of NSCLC primary tumors. Hence, direct disruption of this target through monoclonal antibodies, such as daratumumab, might be effective in patients with NSCLC with CD38 overexpression [48].

Disruption of focal adhesion kinase (FAK), a nonreceptor tyrosine kinase that is frequently amplified in lung cancer cell lines, results in DNA damage and sensitivity to ionizing radiation. In addition, using CRISPR/Cas9 approaches, the presence of FAK was shown to be crucial for the oncogenic and clonogenic abilities of KRAS mutants in tumor xenografts [49,50]. In addition, FAK is significantly overexpressed in patients with NSCLC and is associated with poorer clinical outcomes [51–53], which make it an attractive target to treat NSCLC and prevent distant metastasis.

TAZ is a coactivator of the Hippo pathway and is upregulated in lung cancer. Dual inactivation of TAZ and YAP (a transcriptional



activator) suppressed cell proliferation as well as cancer stem cell (CSC) sphere formation in lung cancer, suggesting them as potential molecular targets [54]. CRISPR-based disruption of oncogenic MUC1-C hindered the growth of KRAS-dependent lung adenocarcinoma cells (i.e., A549 and A460) [55]. In addition, overexpression of MUC1 has been shown in >80% of NSCLCs and is associated with poor prognosis [56,57]. It also has a role in epithelial-mesenchymal transition (EMT) and self-renewal ability, each of which are drug resistance mechanisms in cancer [58,59]. Thus, suppression of MUC1-C might delay resistance or even prevent tumor recurrence. Likewise, it has been reported that overexpression of TNC, an extracellular matrix (ECM) protein, is associated with lung cancer recurrence [60]. CRISPR-based transcriptional activation of *Tnc* led to metastatic dissemination of lung adenocarcinoma cells *in vivo*. This highlights the central role of EMC-related proteins in metastasis and their potential use as recurrence and metastasis biomarkers as well as therapeutic targets in NSCLCs [61].

Chromatin-remodeling genes are frequently mutated, mostly inactivating mutations, in lung adenocarcinoma [62–64]. A recent study used CRISPR technology to knockout *Smrca4*, *Arid1a*, or *Setd2* to investigate their role in lung tumorigenesis. Loss of *Arid1a* and *Setd2* resulted in the development of higher-grade tumors and strong tumor progression in both early- and late-stage lesions, respectively. By contrast, ablation of *Smrca4* led to tumor development, whereas it attenuated disease progression *in vivo* over time [34]. Previously, it was shown that SMARCA4 inactivation promotes NSCLC aggressiveness [65]. Nevertheless, loss-of-function mutations in *SMARCA4* have been reported to increase tumor cell sensitivity to the Aurora kinase A inhibitor VX-680 both *in vitro* and in xenograft mouse models [66]. These discrepancies make it challenging to decide whether SMARCA4 is an appropriate molecular candidate for therapy. However, genetic disruption or chemical-based inhibition of SMARCA4 could be of benefit for patients with more advanced NSCLC.

miRNAs have important roles in cells and their dysregulation has been shown in various types of cancer [67]. A recent study exploited CRISPR/Cas9-based gene activation technology to investigate the role of miRNAs located on 14q32 in lung cancer cells. Overexpression of those miRNAs significantly elevated cell migration and invasion. Moreover, higher expression levels of mir-323b, mir-487a, and mir-539 were associated with metastasis and poorer prognosis in patients with lung adenocarcinoma, especially in those who had never smoked [68]. Thus, these 14q32 miRNAs might be potential targets to prevent tumor cell dissemination and distant metastasis in patients with lung adenocarcinoma.

Overall, application of the CRISPR/Cas9 genome-editing system in lung cancer has led to the identification of several potential therapeutic targets. Both *in vitro* and *in vivo* studies have shown promising results, especially in the suppression of distant metastasis, which is the main cause of death of patients. In addition, CRISPR/Cas9 can target specific oncogenic alleles of certain genes, such as *EGFR*, which is an important step towards cancer gene therapy.

### Breast cancer

Breast cancer is the most common type of cancer and the leading cause of cancer-related death in women worldwide [69]. It is divided into various subtypes with distinct morphologies. Based

on the expression of estrogen receptor (ER), progesterone receptor (PR), ERBB2 (HER2), p53, and Ki-67, it can be classified into four main molecular subtypes: triple-negative/basal-like; the Her2-enriched; luminal A; and luminal B [70]. ER-positive luminal subtypes are the most common types of breast cancer (almost 70%) and resistance to endocrine therapies occurs in ~30% of these patients [71]. Therefore, finding new treatment options is crucial, especially in the case of recurrence. Recent CRISPR-mediated studies have led to the identification of potential therapeutic targets in different breast cancer subtypes.

Distant metastasis is one of the main characteristics of late-stage cancers and the main reason for cancer mortality. Identification of new therapeutic targets could help to prolong patient survival and improve their life quality. MLK3 is a member of the MAP3K family, which is involved in signal transduction and activation of the MAPK pathway. Abrogation of *MLK3* in murine triple negative breast cancer (TNBC) 4T1 cells, which are highly metastatic, led to suppression of cell invasion and migration [72]. In another study, CRISPR-mediated depletion of CX3CR1, a protein involved in the dissemination of tumor cells into blood vessels, in breast cancer cells impaired lodging of the cancer cells to bone and led to a reduction in the number of cancerous lesions in mice [73].

In a recent study by Liao and colleagues, deletion of *Ubr5*, a member of the E3 ligase family, in a murine mammary TNBC model resulted in the inhibition of tumor growth and distant metastasis *in vivo* as well as the promotion of apoptosis and necrosis through impairment of angiogenesis. The authors also showed high expression levels of UBR5 in patients with TNBC, which make this protein interesting for further investigation for targeted therapy [74]. CRISPR-mediated knockout of *CXCR2* (IL-8 receptor) in breast cancer cells, showed a significant reduction in cell migration *in vitro* as well as a lower rate of lung metastasis *in vivo* [75]. *CXCR2* is a stem-like cell marker for TNBC and shows significantly lower expression in this subtype compared with non-TNBC [76]. It is also known that targeting *CXCR2* improves the chemotherapeutic response in lung cancer [77]. Thus, treatment of patients with advanced non-TNBC with anti-*CXCR2* drugs might be beneficial. In addition, *MARK4* and *FERMT2* are other potential targets related to breast cancer cell migration and metastasis that have been identified by CRISPR/Cas9 [78–80].

Several research groups have functionally studied different proteins with oncogenic effects that might be suitable for further investigation as therapeutic targets in breast cancer. For instance, microtubule-associated serine/threonine kinase-like (MASTL) is involved in the DNA damage response and its overexpression correlates with poor clinical outcomes in ER-positive breast cancer [81,82]. CRISPR-based disruption of MASTL kinase reduced cell proliferation in breast cancer cell lines and showed therapeutic effects *in vivo*. Further MASTL expression analysis in human breast primary tumors showed higher expression in tumor cells compared with normal tissue. In addition, higher MASTL expression was significantly associated with poorer prognosis and its abundance was associated with higher histological grades, suggesting its crucial role in the progression of breast cancer [83]. Hence, inhibition of MASTL might suppress tumor growth in high-grade breast tumors.

One study showed that the SRC family kinase (SFK) FYN and protein tyrosine phosphatase N23 (PTPN23) have an important

role in breast cancer. Double knockout of *PTPN23* and *FYN* using CRISPR/Cas9 significantly attenuated cell growth in both Cal-51 cells (a TNBC cell line) and xenograft mouse models. The authors also found that expression of *PTPN23* was positively associated with a better clinical outcome [84]. Moreover, *FYN* is implicated in drug resistance and is highly expressed in resistant cell lines compared with parental cells [85,86]. Thus, inhibition of *FYN* in resistant patients with *PTPN23* loss might be a suitable option to improve clinical outcomes.

Loss of cyclin-dependent kinase 7 (CDK7) in different TNBC cell lines substantially reduced cancer cell growth in these cells, but not in non-TNBC cells [87]. However, elevated expression of CDK7 has been shown in primary breast cancer tissues and its expression is negatively associated with tumor grade and size. In addition, higher expression of CDK7 was associated with better outcome [88]. There are some discrepancies between *in vivo* and patient-based studies, which might be the result of differences in breast cancer subtypes. Nevertheless, CDK7 might be a suitable target at least for a subset of patients with breast cancer. Disruption of *SHCBP1*, a member of the Src homolog and collagen homolog family, in breast cancer cell lines inhibited cell proliferation and promoted apoptosis. The authors also showed that *SHCBP1* is overexpressed in >60% of breast primary tumors and is associated with poorer survival [89]. Thus, suppression of these proteins might result in the inhibition of tumor growth in patients and improve their survival.

Zheng *et al.* performed CRISPR screening for RNA-binding proteins (RBP) involved in breast tumorigenesis. They showed that PHD finger protein 5A (PHF5A) is a key splicing factor and its knockout leads to suppression of cell proliferation, tumor formation, and cell migration in breast cancer cells. They also identified an elevated expression level of PHF5A in primary breast cancer samples and showed its inverse correlation with patient survival [69]. This is similar to a recent report in primary lung adenocarcinoma [90]. Interestingly, inhibition of PHF5A led to tumor growth suppression in glioblastoma xenograft mice [91]. Thus, PHF5A might be a suitable therapeutic candidate not only in breast tumors, but also other types of cancer.

ncRNAs are also important in carcinogenesis and their dysregulation can lead to cancer. Singh *et al.* revealed higher expression of BC200, a long ncRNA (lncRNA) that regulates protein synthesis, in ER-positive breast cancer primary tumors compared with ER-negative ones. Genetic abrogation of BC200 *in vitro* (MCF7 cell line) and *in vivo* led to reduced cell growth through expression of Bcl-x, a proapoptotic protein [92]. In addition, ablation of miR-10b significantly suppressed cell migration in metastatic breast cancer cells (i.e., MDA-MB-231) [93]. These data suggest that genetic suppression of specific ncRNAs with a role in important signaling pathways in tumor cells is a good therapeutic option for the treatment of breast cancer.

In general, breast cancer preclinical studies have led to the identification of several genes and proteins, the inhibition of which might significantly improve treatment outcomes.

### Gliomas

Malignant glioma is the most frequent form of brain primary tumors. Based on WHO guidance, it is classified into astrocytomas, ependymomas, oligoastrocytomas, and oligodendrogliomas. The

grade of malignancy varies from one subtype to another. For instance, oligodendrogliomas and oligoastrocytomas are categorized as grade II and III, respectively, whereas astrocytomas comprise four different grades (I–IV) [94]. Astrocytomas grade IV are also called glioblastoma multiforme (GBM), and accounts for 60–70% of all malignant gliomas [94,95]. GBM is one of the most heterogeneous and aggressive forms of brain tumor, with a poor prognosis [96]. These characteristics might increase chance of recurrence in patients with GBM. Currently, surgery, radiotherapy, and chemotherapy are the common treatment options for GBM [97]. However, different signal transductions and TKIs are now being evaluated in clinical trials.

There are several preclinical studies investigating different putative therapeutic targets in glioma. For example, patients with GBM with *EGFR* mutations and amplifications have poorer prognosis compared with patients with wild-type *EGFR*. Knockout of either wild-type or mutant (EGFRvIII) *EGFR* resulted in the inhibition of tumor growth in human glioma U87 and LN229 cells as well as in mice [98]. Thus, complete inhibition of *EGFR* could be a promising therapeutic option for patients with GBM. However, more detailed studies and confirmations are needed.

Targeting key players of any activated pathway in cancers is predicted to have inhibitory effects on tumor cells. Based on the pathway, it might influence cell proliferation, invasion, and migration abilities. STAT3 activation, ranging from 9% to 83%, has been reported in patients with glioma, probably because of differences in tumor grade [99,100]. A positive correlation has been shown between STAT3 activation levels and glioma grades, suggesting a crucial role for this protein in tumor invasiveness and possibly distant metastasis [101]. Inducing CRISPR-mediated loss-of-function mutations in *STAT3* strongly inhibited GBM tumorigenesis *in vivo* in mice. Moreover, glioma-initiating cells (GICs) were highly addicted to STAT3 and were not viable upon STAT3 depletion [102]. A recent preclinical study also showed that the AK2/STAT3 inhibitor pacritinib strongly inhibited patient-derived GBM cells [103]. Based on these results, inactivation or inhibition of STAT3 by either gene manipulation or chemical compounds, especially during early disease stages, could be beneficial for patients with GBM.

Liu *et al.* investigated the role of ER $\beta$  isoforms in the progression of GBM using CRISPR-mediated knockout in patient-derived GBM cells. Disruption of ER $\beta$  increased migratory and invasive properties of the glioma cells. The authors revealed tumor suppressor activity of ER $\beta$ 1 in GBM cells, whereas ER $\beta$ 5 had more oncogenic effects and its restoration increased cell viability. They also identified significantly higher expression of ER $\beta$ 5 in glioma tumors compared with normal brain tissues, with the highest expression in GBM [104]. Thus, anti-ER $\beta$ 5 drugs could be prescribed as a therapeutic strategy in patients with high-grade glioma in the future.

The CRISPR/Cas9 knockout system has frequently been used to elucidate the role of different proteins in glioma pathogenesis. Peng and colleagues investigated the role of chromatin assembly factor 1 subunit A (*CHAF1A*) in glioma cell survival. Loss of *CHAF1A* significantly increased apoptosis and caused cell cycle arrest in two GBM cell lines (U251 and U87). They also observed that expression of *CHAF1A* was negatively associated with overall survival of patients with GBM [105], similar to a previous study on

colon cancer [106]. Therefore, suppression of CHAF1A in patients might prolong their overall survival. Disruption of brain lipid-binding protein (BLBP) resulted in the inhibition of cell proliferation in U251 GBM cells. Furthermore, higher expression of BLBP was associated with poorer clinical outcome in patients with GBM [107]. In another study, depletion of polo-like kinase 1 (*PLK1*) in mouse model brain tumors improved survival and significantly inhibited tumor growth [108]. Interestingly, several *PLK1* inhibitors, including BI-2536, GSK461364, and volasertib, are currently in clinical trials for the treatment of other solid tumors, but not gliomas [109]. Inhibition of all the genes and proteins discussed could be beneficial for patients and improve clinical outcomes. However, further preclinical and clinical studies are warranted before such approaches enter the clinic.

There are also some reports showing that loss-of-function changes in several ncRNAs can be a promising treatment approach in patients with GBM. CRISPR-mediated knockout of the lncRNA MANTIS resulted in an ~50% reduction in angiogenic capacity of the endothelial cells isolated from GBM and attenuated tube formation. These data showed that the angiogenic capacity of endothelial cells is dependent on MANTIS [110], which could be exploited to impair angiogenesis in tumors. In addition, disruption of miR-10b in GBM cells revealed their strong addiction to this miRNA. It impaired cell viability and resulted in the upregulation of miR-10b verified targets, including cell-cycle inhibitor *P21* and the mediator of apoptosis *BIM*. Disruption of miR-10b was lethal both *in vitro* and *in vivo*. Interestingly, miR-10b was overexpressed in patients with GBM, which makes it a suitable target in gliomas [93]. Thus, suppression of such ncRNAs with oncogenic activity could significantly inhibit tumor growth in patients with glioma.

As discussed above, CRISPR-based preclinical studies have shown promising results in the treatment of gliomas. Several genes, including those encoding kinases, have been shown to have oncogenic roles in glioma, which could be exploited to target tumor cells in a subset of patients. However, further clinical studies are needed to validate these candidate targets.

### Liver cancer

Primary liver cancer is the second cause of cancer-related death in the world and is more common in men. Primary liver cancer is divided into two main subtypes: hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). HCC and ICC account for ~80% and ~15% of the total cases, respectively. The remaining 5% are the rare subtypes of liver cancer [111]. Most patients with liver cancer have a poor prognosis, frequent recurrences, and limited treatment options. Therefore, CRISPR/Cas9 technology could be a promising approach to identify new therapeutic targets in this cancer [112].

CXC chemokine receptor 4 (*CXCR4*) expression levels have significant negative correlation with clinical outcomes in patients with HCC [113]. CRISPR-mediated ablation of *CXCR4* in HepG2 cells resulted in suppressed cell proliferation and migration. Moreover, *CXCR4* attenuation resulted in a smaller tumor size *in vivo* [114]. Thus, *CXCR4* has an oncogenic role in HCC and could be used as a treatment target to control growth or distant metastasis.

Several other potential targets in HCC have been investigated by using CRISPR/Cas9. For example, simultaneous CRISPR-based knockout of *Pten* and overexpression of *Nras* led to HCC formation

*in vivo*, whereas single gene manipulation did not result in tumorigenesis. Aberration in the expression of *Pten* or *Nras* is necessary for liver tumorigenesis, but it is not sufficient if these changes are not present simultaneously [115]. Therefore, inhibition of overexpressed *NRAS* in patients with *PTEN* loss might suppress tumor growth and improve patient survival a subset of HCCs. Nuclear receptor coactivator 5 (*NCOA5*) encodes a coregulator for ERs and is dysregulated in several types of cancer. Genetic knockout of *NCOA5* significantly inhibited cell growth and migration in HCC cells. In addition, loss of the *NCOA5* protein substantially suppressed EMT, which is a common drug resistance mechanism in different cancer types [116]. Nogo-B is a negative regulator of apoptosis [117] and its CRISPR-based deletion led to significant inhibition of cell proliferation *in vitro* as well as suppression of tumor growth and distant metastasis *in vivo* [118]. All the aforementioned genes could be considered as appropriate therapeutic candidates for further *in vivo* studies or clinical trials as inhibitors are developed.

Hepatitis B virus (HBV) is one of the pathogens for HCC, and HBV infection can lead to HCC. One of the potential therapeutic applications of CRISPR/Cas9 system is to prevent HBV-derived liver cancer by destroying viral DNA. HBV particles contain relaxed circular DNA (rcDNA), which is converted to covalently closed circular DNA (cccDNA) upon entering hepatocytes. cccDNA serves as template for all HBV transcripts. Thus, elimination of cccDNA, for instance by cleaving, could cure HBV. Several preclinical studies targeting HBV have shown promising results that might be beneficial to eradicate HBV as one of the main risk factors for liver cancer. Lin and colleagues used CRISPR/Cas9 technology against HBV. Using a gRNA targeting a conserved HBV genomic region, production of EBV surface and core proteins was successfully suppressed both *in vitro* and *in vivo*. The authors also efficiently reduced rcDNA and cccDNA levels in the cells [119]. Another research group showed that HBV-specific gRNA combinations reduced HBV DNA and cccDNA up to 1000- and tenfold *in vitro*, respectively [120]. Li *et al.* used the CRISPR/Cas9 system to successfully eradicate HBV infection from HBV-positive HepG2 cells by deleting the entire integrated HBV genome and disruption of cccDNA [121]. Similar results were reported by other groups [122–124]. In addition, CRISPR-based ablation of flap structure-specific endonuclease 1 (*FEN1*) in Hep38.7-Tet, an *in vitro* HBV model, resulted in reduced cccDNA levels, suggesting a crucial role for the host *FEN1* protein in cccDNA production [125].

### Colorectal cancer

Colorectal cancer is one of the most frequent types of cancer and the fourth cause of cancer-related death worldwide [126]. Adenocarcinoma (ADC) accounts for >90% of all colorectal carcinomas. The remaining 10% are rare subtypes, including adenosquamous, squamous cell, neuroendocrine, spindle cell, and undifferentiated carcinomas. Approximately 95% of colorectal cases are sporadic and 5% are inherited. Colorectal tumors can be triggered by driver mutations in well-known cancer genes, such as *KRAS* and *BRAF*, in ~40% and ~10% of patients, respectively. Treatment of patients is based on histology and molecular testing, but, similar to other cancers, tumors eventually become resistant to treatment [127]. Therefore, overcoming resistance by the identification of new therapeutic targets is crucial.



Yau *et al.* used genome-wide CRISPR/Cas9 screening of *KRAS*<sup>mut</sup> and *KRAS*<sup>wt</sup> colorectal cancer xenografts to identify genes that promote or suppress tumor growth *in vivo*. They showed that disruption of NAD kinase (*NADK*) or ketohexokinase (*KHK*) significantly inhibited *KRAS*<sup>mut</sup> tumor xenografts compared with *KRAS*<sup>wt</sup> xenografts, suggesting them as potential therapeutic targets in *KRAS*-driven colorectal tumors [128]. Several *NADK* and *NHK* inhibitors have been synthesized [129,130]. However, preclinical and clinical studies are needed to evaluate their efficacy and toxicity in human cells.

Caspase-3 (*CASP3*) is involved in apoptosis and its higher activity is associated with a higher risk of tumor relapse in colon cancer [131]. It is overexpressed in colorectal cancer primary tissues and its higher expression is significantly associated with shorter overall survival [132]. CRISPR-mediated knockout of *CASP3* in a colon cancer cell line (HCT116) significantly attenuated cell tumorigenesis, invasion, and migration. Interestingly, deletion of *CASP3* resulted in the higher sensitivity of the cells to radiotherapy both *in vitro* and *in vivo* [133]. Together, these data suggest that inhibition of caspase-3 could be used to improve clinical outcome in patients with colon cancer.

*NAT1* is a member of the *N*-acetyltransferases family and is associated with cancer cell proliferation [134]. In addition, *NAT1* can protect colon cancer cells during nutrient deprivation, and its absence is lethal [135,136]. Genetic ablation of *NAT1* in HT-29 colon cancer cells suppressed cell growth and promoted apoptosis under glucose starvation by enhanced production of reactive oxygen species (ROS) [134]. Thus, inhibition of *NAT1* in combination with specific diets could be an effective method to treat colon cancer. A study by Nishi *et al.* revealed that production of ROS significantly reduced expression of a protein called NHL-repeat-containing protein 2 (*NHLRC2*), resulting in increased levels of apoptosis in HCT116 colon cancer cells. They showed that genetically engineered *NHLRC2*<sup>-/-</sup> cells were more susceptible to ROS-induced apoptosis [137], which might have potential therapeutic benefits for patients with colorectal cancer.

Dysregulation in autophagy is known to be involved in cancer pathogenesis [138]. For instance, *MARCH2* is involved in the regulation of autophagy [139]. CRISPR/Cas9-based disruption of *MARCH2* attenuated cell proliferation, while promoting apoptosis and cell cycle arrest in colon cancer cells both *in vitro* and *in vivo*. Further investigations on primary tumor samples revealed higher expression of *MARCH2* compared with normal tissue. Moreover, higher expression of this protein was significantly associated with poorer overall survival in patients [140]. Therefore, either genetic disruption or chemical inhibition of *MARCH2* could improve clinical outcomes in a subset of patients with colon cancer.

Application of CRISPR/Cas9 genome-editing technology has led to the identification of several other candidate targets in colorectal cancer. For example, deletion of the RNA-binding protein *ELAVL1* (*HuR*) in colorectal cancer cells resulted in a significant reduction in tumor growth compared with the control group *in vivo*. The authors also revealed that *ELAVL1* is substantially overexpressed in colon primary tumors, which makes it a suitable therapeutic candidate [141]. Wu *et al.* knocked out *RBX2*, a component of E3 ubiquitin ligases, in two colon cancer cell lines to investigate its role in tumorigenesis. *RBX2*<sup>-/-</sup> cells showed significantly reduced colony formation and migration capacity *in vitro*. In addition, abrogation of *RBX2* significantly reduced tumor size and lung metastasis *in vivo*.

Further investigations using colorectal primary tumors revealed a higher expression of this protein compared with normal tissues [142]. Thus, *RBX2* appears to be a suitable target in patients with overexpressed *RBX2* to prevent, or at least delay, distant metastasis, which is the main cause of death in all cancer types.

Similar to other types of cancer, ncRNAs have an important role in colon tumorigenesis. It has been demonstrated that a small nuclear RNA, called *SNORA21*, has an oncogenic role in colorectal cancer. CRISPR-based ablation of *SNORA21* resulted in substantial suppression of cell proliferation, invasion, and tumor growth capacities both *in vitro* and *in vivo*. The authors also revealed a significantly higher expression of *SNORA21* in primary colon tumors compared with normal tissues. In addition, they showed that higher expression of *SNORA21* was negatively correlated with overall survival, disease stage and distance metastasis [143]. Thus, *SNORA21* could be a potential therapeutic target and its inhibition could be beneficial for patients with high-stage colorectal tumors.

### CRISPR/Cas9 delivery to cancer cells

Delivery of nucleases, such as Cas9, ZFN, and TALENs, remains one of the most substantial challenges to the use of such therapy in the clinic. With rapid developments of CRISPR-based techniques, it is crucial to develop more efficient, precise, and accurate delivery methods. All nucleases, including the meganucleases, Cas9, ZFN, and TALENs, are biomacromolecules and have similar challenges to their delivery into human cells. Despite different components and characteristics, all nucleases can be generally delivered in the format of DNA, mRNA, or protein. The only difference in the CRISPR/Cas9 system is that the gRNA should be delivered as DNA or RNA molecules compared with the other nucleases [144–146].

There are two types of gene delivery system: viral and nonviral vectors. For basic research, for instance, the identification of gene function and identification of novel therapeutic targets, diverse categories of viral or nonviral delivery methods can be used. Recently, nonviral delivery techniques, such as electroporation, hydrodynamic injection, microinjection, and self-assembled nanoparticles (NPs), were used in *ex vivo* gene-editing using nucleases [147,148]. Yet, *ex vivo* gene editing with NPs and integration of a viral system cannot be used in most tissues types because of low efficiency and safety concerns [149].

Viral vectors are more effective and attractive for the delivery of CRISPR/Cas9 *in vivo*. Compared with traditional non-integrated viral-based gene therapy, in which continuous transgene expression is needed by repeated dose, permanent genome editing can be achieved by transient expression of CRISPR/Cas9 with a single administration. Given that CRISPR/Cas9 has shown high levels of efficiency, specificity, and stability, requirements of the viral vectors for delivery of CRISPR/Cas9 might not be as strict as previously required. The preferred viruses for the delivery of nucleases are AdVs, integrase-defective lentiviruses (IDLV), and adeno-associated viruses (AAVs) [150–152]. Here, we provide an overview of appropriate viral and nonviral vectors used for the delivery of nucleases in cancer cells.

#### Viral vectors

##### AdVs and AAV vectors

AdV and AAV vectors have been extensively studied for the delivery of different nucleases *in vitro*, *ex vivo*, and *in vivo* because of their high

titer, relatively mild immune response, a broad range of cell infectivity, and safety. One study showed that ZFNs can be expressed more efficiently using AdV vectors compared with IDLV in genetically engineered primary T cells [153]. More importantly, modified AdV vectors showed more accuracy and specificity than IDLV for donor DNA delivery [153]. However, host inflammatory responses, including cytotoxic T cells, limits AdV vector application [154]. The other drawback is the expression level of coxsackie-adenovirus receptor (CAR) on the tumor cell surface, which determines the efficiency of delivery using AdV vectors. The CAR expression level has a negative correlation with tumor grade. Thus, AdV is not efficient for the delivery of nucleases directly to high-grade tumors.

Recombinant AAVs (rAAVs) are also attractive options for the delivery of nucleases. They are commonly used in the delivery of ZFNs, TALENs, and CRISPR/Cas9, because AAV vectors have the ability to avoid random integration and show low immunogenic characteristics [152,155–158]. The low packaging capacity (~4.7 kbp) is the main limitation of AAV vectors. Therefore, the recombinant sequence needs to be carefully designed to match the AAV capacity.

### LV and IDLV vectors

Self-inactivating lentiviral vectors (LVs) are commonly used as vehicles for gene therapy in dividing and nondividing cells [159–161]. After decades of preclinical and clinical testing, LVs are being used to treat a variety of genetic diseases, such as X-linked adrenoleukodystrophy and Wiskott–Aldrich syndrome [160,162–164]. Currently, several studies have shown that LVs can be used to efficiently deliver CRISPR/Cas9 to mammalian cells and mice for cancer gene therapy [165–167]. However, unlike using LVs for gene replacement therapy, the continuous expression of Cas9 is unnecessary and might be a problem.

Compared with integrating LVs, IDLVs minimize proviral integration because of mutations in the integrase protein, which make it more suitable for the delivery of CRISPR/Cas9 [151,166]. IDLVs have been successfully used to deliver CRISPR/Cas9 and its donor template *in vitro*. Approximately 6–11% gene knock-in was achieved in hematopoietic stem and progenitor cells (HSPCs) [168]. IDLVs have also been used to codeliver ZFNs/TALENs and HDR donor templates into a variety of cell types, including primary and stem cells, with a relatively high efficiency (>20%). However, IDLV-mediated gene editing can cause undesired gene modifications, such as epigenetic silencing and the induction of genomic rearrangements, particularly in repetitive sequence loci. Another disadvantage is that IDLV-mediated Cas9 delivery leads to a higher off-target effect in quiescent or slowly dividing cells, such as hepatocytes and neurons [169].

### Nonviral vectors

Nonviral vectors are being rapidly developed and several nonviral vectors, including lipid-based vectors and polymer-based vectors, are widely used in gene therapy [170]. Unlike viral vectors, the capacity of nonviral vectors is flexible. In addition, it appears that many disadvantages of viral vectors, such as mutagenesis and immunogenicity, can be addressed by using nonviral vectors [170–172]. Lower immunity or absence of pre-existing immunity in patients is an important advantage of nonviral vectors over viral vectors. However, compared with viral vectors, some nonviral vectors are not able to penetrate cells efficiently, resulting in

low levels of gene transfer efficiency and high *in vivo* toxicity, which could limit their use of in clinical trials [16].

In recent years, nonviral vectors have been used for CRISPR/Cas9 delivery *in vitro* and *in vivo*. For instance, one study showed that up to 80% gene modification can be achieved *in vitro* using cationic liposomes. In addition, cationic liposomes have been successfully used to deliver CRISPR/Cas9 to the inner ear in mouse models [172]. Another study successfully used polyethylenimine (PEI) for the delivery of CRISPR/Cas9 to mouse brain [173].

### Combined viral and nonviral delivery

A recent study showed that the combined use of viral and nonviral vectors (lipid-based vectors and AAVs) achieved >6% gene repair (HDR) in hepatic cells [174]. They also applied this approach to a mouse model and successfully repaired the disease-causing gene. This method might be an efficient option for the delivery of CRISPR/Cas9 to patients.

### Tissue- or tumor-specific delivery of CRISPR/Cas9

Several studies have shown that CRISPR/Cas9 is capable of editing genes with high accuracy and efficiency [19,175,176]. However, for cancer gene therapy, how to specifically deliver CRISPR/Cas9 to cancer cells is still a major obstacle. Herein, we briefly explain some of these delivery methods, including the use of appropriate viral serotypes, specific promoters, and bispecific conjugates.

More than 200 AAV serotypes have been identified so far. Packaging CRISPR/Cas9 into the appropriate AAV serotype for tissue-specific delivery is a promising strategy for cancer gene therapy. Several rAAV vectors have been developed for clinical trials and, recently, the first gene therapy for treatment of an inherited retinal disease using AAV was approved by the US Food and Drug Administration (FDA) [177]. One study showed that CRISPR/Cas9 delivery using AAV serotype 9 (AAV9) was highly efficient for tissue-specific delivery and did not cause substantial cellular damage *in vivo* [178].

Utilizing a promoter-based ‘AND’ logic gate can control CRISPR/Cas9 to be specifically expressed in bladder cancer cells [179]. The strategy of promoter-based logic gates, including ‘AND’, ‘OR’, and ‘NOT’, have the potential to create more complex and precise regulatory patterns that can be conditionally expressed. Delivery of CRISPR/Cas9 to tumor cells with such precision could accelerate the use of CRISPR-based cancer gene therapy from bench to bedside.

Low expression of CAR on the tumor cell surface and lack of specific receptors significantly limits the clinical use of AdV vectors in cancer gene therapy. However, redirecting AdV vectors to tumor-specific cell surface target receptors, such as EGFR, EpCAM, CD40, PDGF-Rbeta, and VEGFR, can selectively target tumors, which may improve the specificity and efficiency of AdV simultaneously [180–183].

### CRISPR/Cas9; hopes and challenges in cancer treatment

CRISPR/Cas9 genome-editing technology has shown promising results in preclinical studies. It is a convenient tool that has enabled researchers to perform gene manipulation at a single base-pair resolution in a relatively efficient way. Preclinical studies have led to the identification of several potential therapeutic targets by using CRISPR-based methods (Table 3). This has brought

TABLE 3

List of (potential) therapeutic targets in different solid tumors identified by using CRISPR/Cas9 genome-editing technologies<sup>a</sup>

Target	<i>in vitro</i>	Cell line	<i>in vivo</i>	CRISPR	Vector	Effect	Refs
Lung cancer							
<i>EGFR</i>	+	H1975	+	Knockout of mutant allele (L858R)	AdV	Promoted cell death and reduced tumor size	[4]
<i>EGFR</i>	+	H1975	+	Knockout of mutant allele (L858R)	LV	Reduced cell proliferation and tumor size	[43]
<i>KRAS</i>	+	A549 and KP-Kras <sup>G12D/1</sup> (mouse lung cancer cells)	+	Both alleles knockout	LV	Reduced cell proliferation	[44]
<i>CD38</i>	+	A549	+	Both alleles knockout	Nonviral	Inhibited cell growth and invasion	[48]
<i>FAK</i>	+	H460	+	Both alleles knockout	Nonviral	Reduced cell proliferation and tumor size	[50]
<i>TAZ</i> and <i>YAP</i>	+	A549	+	Both alleles knockout	LV	Dual knockout inhibited cell proliferation, cancer stem cell sphere formation, and tumor formation	[54]
<i>MUC1</i>	+	A549 and H460	—	Both alleles knockout	LV	Suppressed MYC expression and inhibited cell growth	[55]
<i>Tnc</i>	+	Collection of cell lines isolated from mice with primary lung tumors (KP mice)	+	CRISPR-mediated gene activation	LV	Enhanced metastatic potential of cells	[61]
<i>Smarca4</i>	—	—	+	Both alleles knockout	LV	Induced tumor development, but attenuated disease progression <i>in vivo</i> over time	[34]
<i>mir-323b</i> , <i>mir-487a</i> and <i>mir-539</i>	+	H2009	—	CRISPR-mediated gene activation	LV	Promoted cell migration and invasion	[68]
Breast cancer							
<i>Mlk3</i>	+	4T1 (murine breast cancer cells)	+	Both alleles knockout	Nonviral	Suppressed cell invasion and migration	[72]
<i>CX3CR1</i>	+	MDA-MB-231	+	CRISPR-based transcriptional suppression (CRISPR interference)	LV	Impaired lodging of cancer cells to mouse bone and reduced number of cancerous lesions in each animal	[73]
<i>Ubr5</i>	+	4T1 (murine breast cancer cells)	+	Both alleles knockout	Nonviral	Promoted apoptosis and inhibited tumor growth and distant metastasis	[74]
<i>CXCR2</i>	+	MDA-MB-231	+	Both alleles knockout	Nonviral	Inhibited cell proliferation, migration, tumor size, and rate of lung metastasis	[75]
<i>MARK4</i>	+	MDA-MB-231	—	Both alleles knockout	Nonviral	Inhibited cell proliferation and migration	[79]
<i>FERMT2</i>	+	MDA-MB-231 and 4T1 (murine breast cancer cells)	+	Both alleles knockout	LV	Inhibited cell migration, invasion, and tumor growth	[80]
<i>MASTL</i>	+	MDA-MB-231, MCF7, BT549, and BT-483	+	Both alleles knockout	LV	Impaired cell proliferation and tumor growth	[83]
<i>PTPN23</i> and <i>FYN</i>	+	Cal-51	+	Both alleles knockout	LV	Dual knockout inhibited cell proliferation and tumor growth	[84]
<i>CDK7</i>	+	MDA-MB-231, MDA-MB-468, HCC38, BT549, and SUM149	+	Both alleles knockout	LV	Inhibited cell proliferation and impaired cell viability	[87]
<i>SHCBP1</i>	+	MDA-MB-231 and MCF7	—	Both alleles knockout	LV	Inhibited cell proliferation and promoted apoptosis	[89]
<i>PHF5A</i>	+	CA1a and DCIS	+	CRISPR screen targeting RNA-binding proteins	LV	Suppressed cell proliferation, migration, and tumor formation	[69]
<i>Inc BC200</i>	+	MCF7	+	Both alleles knockout	Nonviral	Inhibited cell proliferation and tumor growth	[92]
<i>miR-10b</i>	+	MDA-MB-231	—	Both alleles knockout	LV	Suppressed cell migration	[93]
Glioma							
<i>EGFR</i>	+	U87 and LN229	+	Knockout of both wild-type and mutant (EGFRvIII) EGFR	LV	Inhibited cell proliferation and tumor growth and improved survival	[98]

TABLE 3 (Continued)

Target	<i>in vitro</i>	Cell line	<i>in vivo</i>	CRISPR	Vector	Effect	Refs
<i>STAT3</i>	+	MT330	+	Both alleles knockout	LV	Inhibited tumorigenesis <i>in vivo</i> , but limited effect on cell proliferation <i>in vitro</i>	[102]
<i>ERβ</i>	+	U87 and U251	+	Both alleles knockout of <i>ERβ</i> followed by reintroduction of <i>ERβ1</i> and <i>ERβ5</i> separately	Nonviral/LV	<i>ERβ</i> knockout elevated migratory and invasive properties. Overexpression of <i>ERβ5</i> enhanced cell migration and invasion	[104]
<i>CHAF1A</i>	+	U87 and U251	—	Both alleles knockout	LV	Inhibited cell proliferation, increased apoptosis, and caused cell cycle arrest	[105]
<i>BLBP</i>	+	U251	—	Both alleles knockout	LV	Inhibited cell proliferation and caused cell cycle arrest	[107]
<i>PLK1</i>	NA	NA	+	Both alleles knockout	Nonviral	Inhibited tumor growth and improved survival	[108]
<i>lncRNA MANTIS</i>	<i>Ex vivo</i>	Endothelial cells isolated from GBM	+	Both alleles knockout	Nonviral	Reduced angiogenic capacity of cells and suppressed tube formation	[110]
<i>miR-10b</i>	+	U251, LN229, and A172	+	Both alleles knockout	LV	Impaired cell viability, enhanced expression of <i>miR-10b</i> targets, and suppressed tumor growth	[93]
Liver cancer							
<i>CXCR4</i>	+	HepG2	+	Both alleles knockout	Nonviral	Inhibited cell proliferation and migration and reduced tumor size	[114]
<i>Pten</i> and <i>Nras</i>	—	—	+	Both alleles knockout and CRISPR-mediated gene activation	Nonviral	Simultaneous knockout of <i>Pten</i> and activation of <i>Nras</i> led to liver tumorigenesis	[115]
<i>NCOA5</i>	+	LM3	—	Both alleles knockout	LV	Inhibited cell proliferation and migration, suppressed tumor formation and EMT	[116]
<i>Nogo-B</i>	+	SMMC-7721 and QGY-7703	+	Both alleles knockout	Nonviral	Inhibited cell proliferation and suppressed tumor growth and distant metastasis	[118]
Colorectal cancer							
<i>NADK</i> and <i>NHK</i>	+	HCT116 <i>KRAS</i> <sup>WT/−</sup> , HCT116 <i>KRAS</i> <sup>WT/mut</sup> , DLD-1 <i>KRAS</i> <sup>WT/−</sup> , and DLD-1 <i>KRAS</i> <sup>WT/mut</sup>	+	Genome-wide CRISPR screening knockout	LV	Inhibited <i>KRAS</i> <sup>mut</sup> tumor growth	[128]
<i>CASP3</i>	+	HCT116	+	Both alleles knockout	NA	Suppressed EMT and attenuated cell tumorigenic, invasion, and migration abilities	[133]
<i>NAT1</i>	+	HT-29	—	Both alleles knockout	Nonviral	Inhibited cell growth and promoted apoptosis under glucose starvation	[134]
<i>NHLRC2</i>	+	HCT116	—	Both alleles knockout	LV	Inhibited cell proliferation and promoted apoptosis	[137]
<i>MARCH2</i>	+	HCT116	+	Both alleles knockout	NA	Inhibited cell proliferation, promoted apoptosis and cell cycle arrest, reduced tumor size	[140]
<i>ELAVL1</i>	+	HCT116	+	Both alleles knockout	Nonviral	Suppressed tumor growth	[141]
<i>RBX2</i>	+	HCT116 and SW480	+	Both alleles knockout	Nonviral	Inhibited colony formation and cell migration capacity, reduced tumor size and lung metastasis	[142]
<i>SNORA21</i>	+	HCT116 and SW48	+	Both alleles knockout	LV	Suppressed cell proliferation, invasion and tumor growth	[143]

<sup>a</sup> Abbreviation: NA: not available.

hope for the therapeutic application of this technology in cancer treatment. However, there are some concerns regarding its application in the clinical setting. Here, we have mainly discussed genes with oncogenic activities because gene knockout is more convenient compared with gene knock-in using CRISPR/Cas9 (Fig. 2). In addition, oncogenes are usually overexpressed in primary tumors and are more amenable to pharmacological inhibition.

Although malignant tumors are genetically heterogeneous, tumor bulk is mainly the result of outgrowth of one or two dominant clones [184] caused by driver mutations in certain genes. sgRNAs can distinguish between mutant and wild-type alleles in tumor cells, reducing off-target effects and improving the specificity [185]. Therefore, hotspot driver mutations in genes such as *EGFR*, *KRAS*, *BAP1*, *BRAF*, *BRCA1*, and *BRCA2* can be exploited as a therapeutic



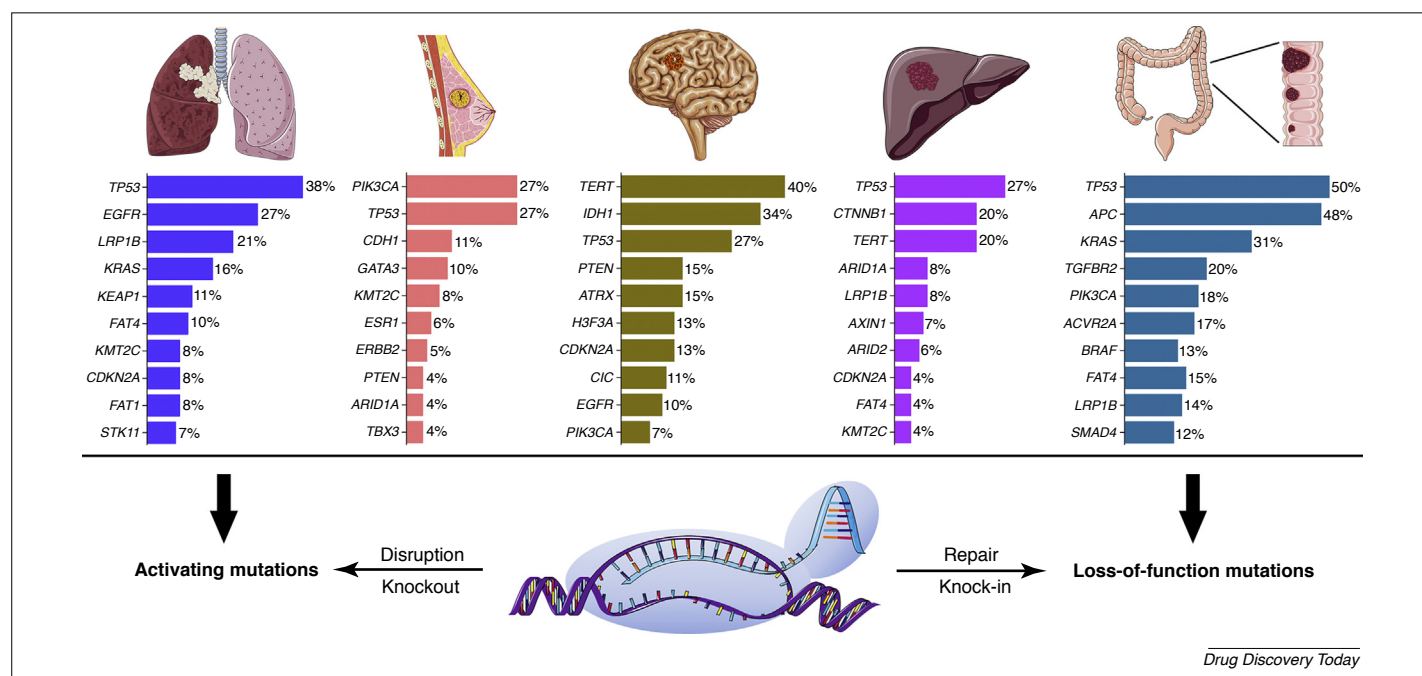


FIGURE 2

Potential therapeutic clustered regularly interspaced short palindromic repeats (CRISPR)-mediated targeting strategies for cancer gene therapy. The priority option for cancer gene therapy is the disruption of oncogenes rather than repairing inactivated tumor suppressor genes. The bar charts show the top-ten mutated genes in different cancers based on the COSMIC database (v87). Mutation frequencies have been calculated using a weighted average mutation frequency based on sample size across all studies. This figure contains modified elements from Servier Medical Art (<http://smart.servier.com>).

approach at least in a subset of patients. One of the main advantages of this strategy is that normal cells, do not contain the mutant alleles, are not targeted and, thus, remain intact. In addition, mutations in oncogenes such as *KRAS* are indicators of drug resistance and poor prognosis in a subgroup of patients with NSCLC, including Asians or women with low-grade tumors [186]. By contrast, CRISPR/Cas9 can precisely target a specific locus in the genome. Therefore, substituting *KRAS* mutant versions (p.G12V and p.G12D) with the wild-type allele could positively improve treatment response in *KRAS*-dependent cancers.

Series of CRISPR-associated nucleases have been discovered over the past few years that can greatly facilitate genome editing. However, there are still some major problems that need to be solved before CRISPR/Cas9 enters the clinic, such as off-target effects [187], continuous activity of Cas9 [187], low efficiency of current delivery systems [187], low efficiency of CRISPR/Cas9-mediated gene knock-in [168], pre-existing adaptive immunity [187], and uncontrollable DNA repair [187]. In addition, recent studies have shown that p53 can inhibit CRISPR/Cas9 gene-editing efficiency [188,189]. According to these studies, CRISPR/Cas9 tends to target cells with intact p53, leaving behind p53-deficient cells, which have the potential to become cancerous. Thus, evaluation of p53 protein before and after gene editing for the treatment of the patients is crucial. However, CRISPR-induced p53 activation appears to occur only during gene editing by HDR. The base editors probably will not trigger p53 and, thus, are safer in this regard compared with CRISPR/Cas9 technology.

Selection of the right target is an important step towards developing a new treatment strategy. A candidate protein and/or molecule cannot be considered as an appropriate target based only on

its higher expression in primary tumor tissues. Extensive functional *in vitro* and *in vivo* studies are required before proceeding to a clinical trial. For example, *FOXC*, a transcription factor, is considered as prognostic biomarker in breast cancer and has been suggested as a therapeutic target [190,191]. However, a recent study by Mott and colleagues showed no difference in tumor size and metastasis between *FOXC1*<sup>-/-</sup> and parental tumor cells *in vivo* [192]. Similarly, one recent thought-provoking study showed that maternal embryonic leucine zipper kinase (*MELK*) is not a cancer target, whereas multiple ongoing clinical trials are trying to inhibit *MELK* to treat cancer [193]. These results highlight the importance of *in vivo* studies using CRISPR/Cas9 in the validation of biomarkers and therapeutic targets and their reliability.

Overall, there are four steps to bring a protein or molecule as a therapeutic approach from the bench to the clinic: (i) identification of key molecules in different diseases; (ii) validation of those molecules by *in vitro* and *in vivo* studies; (iii) development of an efficient method to inhibit that specific molecule; and (iv) successful Phase I–III clinical trials. If any of these steps fails, new therapeutic approaches will not be able to enter into clinics.

The low rate of all-alleles knockout in cancer cells is another important challenge that has to be taken into account. This is caused by the high amount of aneuploidy in cancer cells, which can result in an unpredictable outcome [194]. Therefore, the application of multiple gRNAs for a certain gene can increase the chance of all-alleles knockout in cancer cells. However, this strategy might lead to a subpopulation of cells with active alleles resulting from an in-frame repair of DSB in the target site induced by several gRNAs. In addition, not all gRNAs have the same efficiency; some are more active than others. One solution is to

carefully design and evaluate each gRNA *in vitro* and *in vivo* for certain types of cancer cell and then choose the most efficient combinations. This can reduce the number of gRNAs for a certain gene while increasing the efficiency of the technique. Interestingly, the distance between different gRNAs can also influence the knockout efficiency. It has been shown that multiple carefully designed adjacent gRNAs can improve the chance of having cells with complete gene knockout [195]. In addition, HDR is active in S and G2/M phases, but DSB repairs occur more in G0/G1. Thus, using cytostatic drugs to introduce cell cycle arrest might regulate the repair process in a certain extent [188,189]. In addition, new versions of Cas9, such as Cpf1, are able to generate staggered DSBs, which might result in more accurate and precise repairs [23].

Efficiency of the delivery methods to cancer cells remains one of the biggest challenges in the application of CRISPR/Cas9 as a therapeutic tool in cancer treatment. More specific and efficient delivery vectors need to be developed to achieve sufficient levels of transduction and transgene expression. Traditional virus delivery approaches, such as AdV, LV, and AAV, are currently not sufficient enough to reach clinical requirements for delivery, especially in targeting cancer cells. Therefore, using CRISPR/Cas9 to improve currently available treatment strategies appears more realistic. For instance, engineered universal CAR-T cells by CRISPR/Cas9 can improve antitumor efficacy (Table 1). In addition, CRISPR/Cas9 can be utilized to resensitize drug-resistant tumor cells and improve the treatment response by deleting and/or modifying resistance-related genes. Finally, arming oncolytic viruses with CRISPR/Cas9 could be a new approach to treat cancers [196].

Another attractive application of CRISPR/Cas9 is to improve programmed cell death protein 1 (PD-1)-associated immunotherapies. CRISPR-based disruption of PD-1 in T cells significantly suppresses expression of PD-1 and promotes the cellular immune response *in vitro* [197]. An ongoing Phase I clinical trial in China is using CRISPR-engineered T cells to investigate the safety and efficiency of this immunotherapy-based treatment in patients with metastatic NSCLC [198]. In this method, peripheral T cells of the patients are collected, *PD-1* is knocked out by CRISPR/Cas9, and cells are reinfused to the patients. The engineered T cells escape suppression by PD-L1-positive tumor cells and can perform their antitumor activity [199]. This type of combination therapy, especially combining CRISPR/Cas9 with CAR-T- or PD-1-associated trials (Table 1), could improve outcomes of clinical cancer treatment in the near future.

### Concluding remarks

In conclusion, CRISPR/Cas9 genome-editing technology has led to the identification of several potential therapeutic targets in different malignancies. However, the use of CRISPR/Cas9 as a treatment option in cancer involves several issues, such as minimizing off-target effects, immunological responses to Cas9, validation of therapeutic targets in animal models, and optimization of the delivery methods, which have to be addressed before entering into use in the clinic. Despite all the above-mentioned challenges, CRISPR/Cas9 has brought invaluable opportunities in the field of cancer gene therapy and could have a crucial role in cancer treatment.

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